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Engineered alphavirus replicon vaccines based on known attenuated viral mutants show limited effects on immunogenicity

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ABSTRACT

The immunogenicity of alphavirus replicon vaccines is determined by many factors including the level of antigen expression and induction of innate immune responses. Characterized attenuated alphavirus mutants contain changes to the genomic 5′ UTR and mutations that result in altered non-structural protein cleavage timing leading to altered levels of antigen expression and interferon (IFN) induction. In an attempt to create more potent replicon vaccines, we engineered a panel of Venezuelan equine encephalitis-Sindbis virus chimeric replicons that contained these attenuating mutations. Modified replicons were ranked for antigen expression and IFN induction levels in cell culture and then evaluated in mice. The results of these studies showed that differences in antigen production and IFN induction in vitro did not correlate with large changes in immunogenicity in vivo. These findings indicate that the complex interactions between innate immune response and the replicon's ability to express antigen complicate rational design of more potent alphavirus replicons.

Introduction

Vaccine vectors based on alphaviruses have proven to be potent inducers of antigen-specific immunity (Atkins et al., 2008; Geall et al., 2012; Leitner et al., 2003; Rayner et al., 2002; Ulmer et al., 2012). These vaccines are based on derivatives of the alphavirus genome, which is a message-sense, single-stranded RNA with a 7-methyl-G cap at their 5′ terminus and a poly(A)-tail at their 3′ terminus (Strauss and Strauss, 1994). Alphavirus genomes encode four non-structural proteins, nsP1–4, which are translated directly from the genomic RNA and interact with host factors to form replicative enzyme complexes (Lemm and Rice, 1993; Lemm et al., 1994). These replicative complexes synthesize the negative-strand RNA intermediates, new viral genomes and the subgenomic RNA, which encodes for the structural proteins necessary for viral particles assembly (Strauss and Strauss, 1994).

Alphavirus vaccine vectors have been developed from viral genomes by replacing the structural protein genes with heterologous genes. The resulting RNAs, called replicons, are capable of directing their own replication and express high levels of the heterologous gene when they are introduced into the cytoplasm of host cells. Since these replicons lack the alphavirus structural protein genes, they are incapable of forming virions and spreading to adjacent cells. However, replicons can be efficiently packaged into virus replicon particles (VRPs) by introducing them into cells where the structural proteins are provided in trans (Perri et al., 2003; Pushko et al., 1997).

Several features of VRPs make them attractive candidates for use as vaccine vectors, first of all their ability to stimulate strong immune responses against the encoded antigen. The heterologous gene is expressed at high levels (Xiong et al., 1989) and can be delivered to a variety of cell types, including antigen-presenting cells (Gardner et al., 2000; MacDonald and Johnston, 2000; Nishimoto et al., 2007). The in vivo vector amplification, through double-stranded RNA intermediates, stimulates aspects of innate immunity, such as activation of the type I interferon (IFN) (Leitner et al., 2003). Furthermore, the production of antigen in vivo in a manner that mimics production of antigens by viral pathogens, elicits both humoral and cellular immune responses (Leitner et al., 2003; Perri et al., 2003) that have been shown to confer protection against viral challenge in many animal models (Ryman et al., 2002). The induction of neutralizing antibodies and polyfunctional CD4+ and CD8+ T cell responses has been demonstrated in humans in a clinical trial performed with VRPs encoding cytomegalovirus gB and pp65/IE1 proteins (Bernstein et al., 2009).

The efficient immune responses elicited by alphavirus replicons has been attributed both to the production of high levels of correctly processed heterologous proteins (Liljestrom and Garoff, 1991) and to the activation of innate responses. Upon alphavirus infection, the double-strand RNA (dsRNA) produced by replication

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stimulates the innate immune system by activating conserved receptors, referred to as pattern recognition receptors, that are broadly expressed by immune and non-immune cells and include the RNA sensors PKR, MDA-5, and/or RIG-I. The signals coming from the stimulated receptors activate IRF-3/7-dependent signaling pathways that induce the IFN-mediated antiviral response (Gitlin et al., 2006; Hidmark et al., 2006; Pichlmair et al., 2009; Ryman et al., 2002). IFN signaling is not only a key component of the innate immune response to viral infections (van den Broek et al., 1995), it also plays an important role in potentiating the adaptive immune responses (Fink et al., 2006) and consistent with this latter activity, alphavirus replicon particles have been shown to adjuvant adaptive immune responses of co-administered proteins (Hidmark et al., 2006; Thompson et al., 2006; Thompson et al., 2008a). However, the relative importance of the levels of the expression of replicon-encoded antigens and activation of IFN response by the replicon (which can both help to control replicon replication and adjuvant responses to the heterologous antigen) in the adaptive immune responses induced by the replicon-encoded antigens is unclear.

The viral genome sequences involved in the regulation of the alphavirus RNA replication, subgenomic RNA transcription, and IFN induction have been described for multiple members of the alphavirus family. In the case of the best-studied member of the encephalitic alphaviruses, Venezuelan equine encephalitis virus (VEEV), viral pathogenesis strongly depends on the sequence of the RNA promoter, as demonstrated by studies showing that the 3rd base of the positive-sense viral genome ((+) RNA) controls viral attenuation (Kinney et al., 1993). This mutation, corresponding to a G3→A change (G3A), is found near the end of a 44-base untranslated region found at the 5′ end of the of the viral genome (5′ UTR), which is predicted to form part of a complex secondary structure (Kulasegaran-Shylini et al., 2009a)). The 5′ UTR (more precisely, its complement in the negative-strand of the viral genome (−) RNA) functions as the core promoter for positive-strand genome synthesis (Frolov et al., 2001) and promotes efficient viral genome replication (Gorchakov et al., 2003). As indicated above, the sequence of the 5′ UTR in VEEV plays a significant role in virus pathogenesis, with the G3A mutation, found in the attenuated TC-83 strain of this virus, as one of the major determinants of its less pathogenic phenotype (Kinney et al., 1993; White et al., 2001). Interestingly, the G3A mutation increases transcription of the viral genome, down-regulates transcription of the subgenomic RNA, and results in a decrease in the molar ratio of subgenomic to genomic RNA synthesis (Kulasegaran-Shylini et al., 2009a, 2009b).

In the case of the arthralgic alphaviruses, the cleavage site between nsP1 and nsP2 proteins appears to be a major determinant of viral pathogenesis. A single amino acid mutation of the P3 position of the nsP1/nsP2 cleavage site for two distant related alphaviruses, SINV and Ross River Virus (RRV), resulted in enhanced type I IFN induction and virulence attenuation in comparison to the wild-type (wt) viruses (Cruz et al., 2010). The wt amino acid in the P3 position of the nsP1/nsP2 cleavage recognition domain (nsP1 position 538 in SINV and 532 in RRV) has been shown to be both necessary and sufficient for rapid and efficient inhibition of STAT1 activation (Simmons et al., 2010). When the residue at position P3 is mutated in SINV, the virus is rendered unable to counteract the Jak/STAT signaling necessary for IFN activation of cells, and consequently these mutant viruses are highly attenuated (Simmons et al., 2010).

The aim of this study was to construct alphavirus-based replicon vaccines that were capable of eliciting improved immune responses. We looked to known attenuated viruses as a guide for determining how to alter the interaction between virus and immune system. There are many attenuated alphaviruses that have been characterized to identify the mutations and mechanisms responsible for their attenuation. Many of these attenuated mutants contain changes to their 5′ UTR or non-structural coding regions. Mutations in these regions often simultaneously alter several aspects of the virus lifecycle including replication, protein expression, and virus/host interactions. Due to these pleiotropic effects it is difficult to identify a single phenotypic change that correlates with attenuation but all of the mutant viruses do have an altered interaction with the host immune response and they could possibly lead to altered immune reponses in the context of an alphavirus-based replicon. Therefore we selected a sampling of the known attenuating mutations and built them into our existing replicons to then empirically test them for improved immune responses. We used antigen expression levels and IFN production as tools to characterize the mutant replicons in vitro before testing them in vivo. The replicon we used is derived from VEEV but contains regions from SINV, as the 3′ UTR region. The structural proteins are derived from SINV and to ensure efficient packaging of VEEV replicons into SINV virions, the SINV packaging signal was introduced into the nsp3 region of the VEEV replicon. The resulting VRPs are similar to VEEV VRPs in production yields from electroporated BHK cells, RNA replication and antigen expression in infected cells, interferon resistance, and immunogenicity in vivo (Perri et al., 2003).

Results

Effect of 5′UTR mutations on predicted RNA secondary structures and specific infectivity of the VEEV/SINV replicons

Since the G3A mutation found in TC-83 viral genome is one of the primary determinants of its less pathogenic phenotype, and this attenuation is associated with changes in type I IFN induction, we targeted this site for analysis in our VEEV/SINV replicon. In silico folding of the 5′ ends of the genomes of TC-83 and its parental genome (the Trinidad Donkey strain of VEEV, which is found in the 5′ end of our VEEV/SINV genome) revealed that this change has dramatic effects on the predicted secondary structures (compare G3 and G3A in Fig. 1A) (Kulasegaran-Shylini et al., 2009a; Kulasegaran-Shylini et al., 2009b). In the case of the wt genome (G3), the predicted (−) RNA structure mirrors the stem-loop structure of the positive strand (originally reported by Kulasegaran-Shylini 2009b) (Fig. 1A). Within the 5′ UTR, mutations in position 3 that have been shown to be responsible for the attenuation of vaccine strain TC-83 have a profound effect on the predicted secondary structure of the positive strand (Fig. 1A). Furthermore, this mutation leads to predictions of multiple (−) RNA structures of similar overall free energy suggesting a structure that is more dynamic than that predicted for the parental virus. The most stable predicted conformation is characterized by a longer stem, with two bulges, and a smaller terminal loop (Kulasegaran-Shylini et al., 2009b). To determine if this mutation would also affect our VEEV/SINV replicon (Fig. 1B), we created a G3A variant by using site-specific mutagenesis of the parental G3 replicon and compared infectivity of derivatives expressing either green fluorescent protein (GFP) or secreted alkaline phosphatase (SEAP) in BHK-V cells. Transfection efficiency of in-vitro synthetized RNAs was measured using antigen specific staining by cytofluorimetric (GFP-expressing cells) and colorimetric (SEAP-expressing cells) analysis. The G3A replicon was characterized by more than 2-fold increase in specific infectivity, measured as a number of GFP- or SEAP-positive cells per ng of transfected RNA, over the wt G3 backbone (Fig. 1C). We also observed a slight decrease in antigen expression (data not shown), that, according to

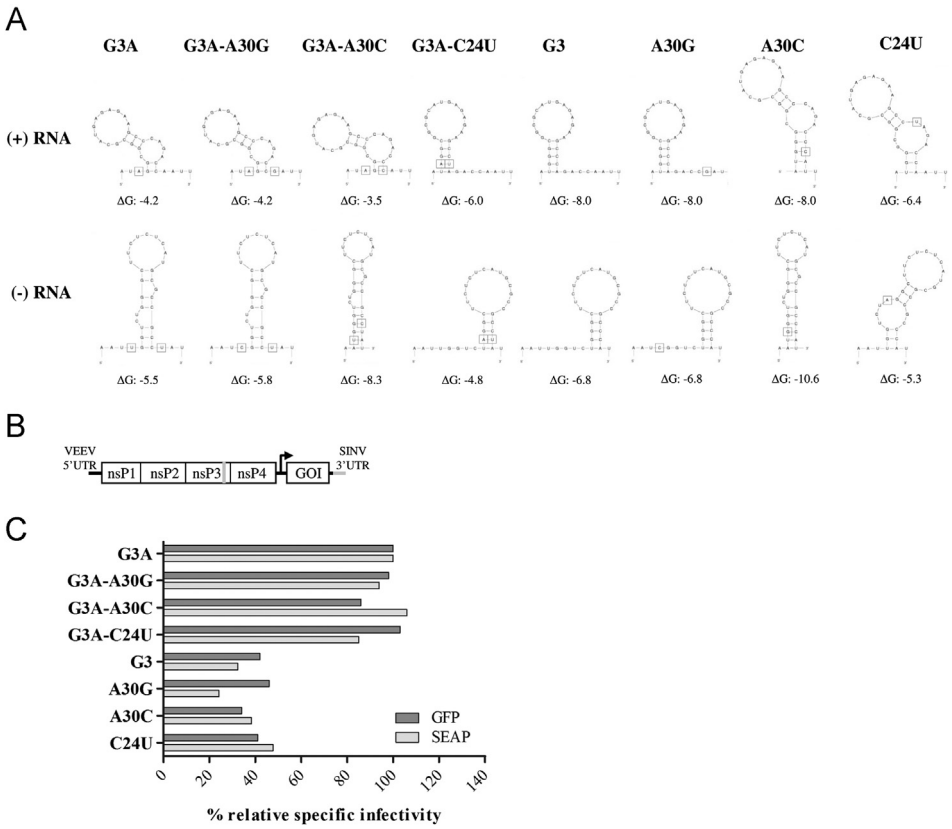

Fig. 1. Effects of 5′ UTR mutations on predicted RNA secondary structures and on VEEV/SINV replicons infectivity. (A) Computer-prediction (M-fold) (Jaeger et al., 1989) of secondary structures of the 5′ termini variants having mutations in position 3, 24 and/or 30, showed a variety of secondary structures both on (+) and (−) RNA strands. The predicted foldings of the wt sequence (G3) are shown. The overall free energy (ΔG, expressed in kcal/mol) is indicated. When multiple structures were predicted for the same sequence, we selected the one having the lowest free energy. (B) Schematic representation of the VEEV/SINV replicon used to test the mutations from (A). The arrow indicates the position of the subgenomic promoter. VEEV 5′ UTR: VEEV wt 5′ UTR sequence having a G in position 3 (G3 wt control); GOI: gene of interest/antigen; nsPs: non-structural proteins; light gray regions indicate sequences from SINV. (C) Analysis of the effect of the 5′ UTR mutations on VEEV/SINV replicon specific infectivity. The mutations from (A) were inserted in the G3 genome by PCR-based mutagenesis. The PCR products were used as template for in vitro transcription and capping. Equal number of BHK-V cells was transfected with 10^9 copies of RNA carrying the indicated 5′ UTR mutation and expressing GFP or SEAP reporter gene. The number of positive cells was evaluated 12 h post-transfection. The shown values are relative to the G3A-specific infectivity.

the data of Kulasegaran-Shylini et al. (2009b), could be the result of the G3A-specific lower subgenomic RNA transcription.

In order to find new genome structures that might alter IFN induction without changes in antigen expression level, we used the data from Kulasegaran-Shylini et al. (2009b), and in silico analyses (Jaeger et al., 1989) to test the potential of different point mutations to produce structures in the (+) and (−) RNA strands that mimicked those found on the G3 (wt) and G3A (TC-83) genomes. Our overall strategy was to select mutations having an A in position 3, since the G3A mutation was associated with higher in vitro specific infectivity, and a folding resembling the conformation of the G3 or G3A on the (−) RNA, as this is the region that acts as a promoter for the synthesis of the (+) RNA. Included in these analyses was the G3A-C24U structure that was previously described by Kulasegaran-Shylini et al. (2009a). This mutant is characterized by the TC-83-like G3A mutation and by a predicted secondary structure that is identical to the wt G3 5′ UTR on both the (+) and (−) RNA strands. To test the importance of the single-strand RNA in the 3′ terminus of the (−) RNA we predicted the secondary structure of 5′UTRs mutated in position 3 and in compensatory positions using in silico tools. Among the variety of possible combinations, we identified and selected the G3A-A30C and G3A-A30G sequences (Fig. 1A), whose predicted structures on the (−) RNA are identical to the G3A one, except for the absence or the presence of the single-strand tail, respectively.

To control and to evaluate the contribution of the G3A mutation on the selected 5′UTRs mutants, we in silico tested the effect of the selected mutations in position 24 and 30 in the G3 (wt) genome, obtaining the C24U, A30C and A30G predicted structures (Fig. 1A). The computer-predicted secondary structures of the (+) and (−) RNA strands of the final panel of mutants selected for further analysis are shown in Fig. 1A.

To test the effect of these 5′UTR modifications on genome replication and antigen expression, the selected mutations were transferred to the VEEV/SINV replicons expressing GFP or SEAP and in vitro transcribed RNAs were transfected into BHK-V cells. The results of these studies are shown in Fig. 1C, where the data are standardized to the specific infectivity of the G3A replicon. These data suggested that the major determinant of specific infectivity is the nucleotide sequence at position #3. All the mutants having a G3A mutation had the same phenotype as the G3A replicon, whereas all of the mutants with the wt G3 showed lower specific infectivity, and the presence of other mutations, which were predicted to alter the secondary structures in one or both strands, had no detectable effect on specific infectivity.

Effect of 5′UTR mutations on expression levels, activation of innate immune response, and immunogenicity of VEEV/SINV replicons

To further dissect the role of sequence and predicted secondary structures of the terminal regions of the (+) and (−) RNA on the replicon phenotype, the G3, G3A, G3A-C24U, and G3A-A30C mutants were selected for further characterization. The G3A-C24U represents an interesting alternative to the current replicon

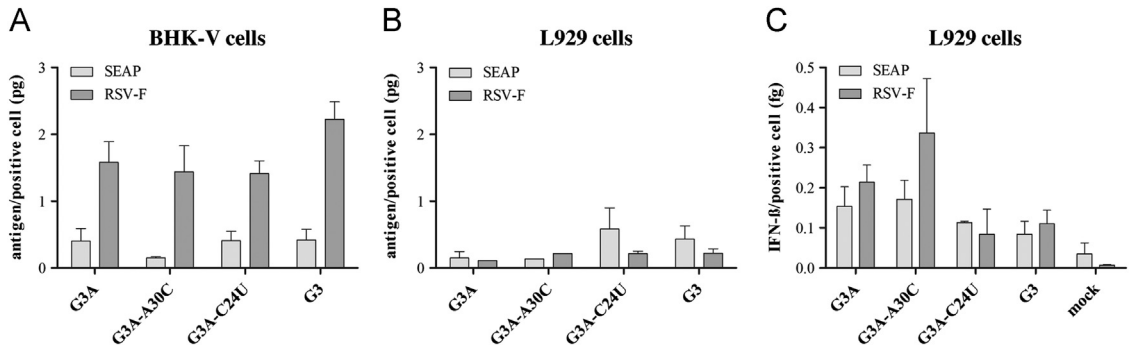

Fig. 2. Effect of 5′ UTR mutations on expression levels and activation of innate immune response of VEEV/SINV replicons. Antigen expression levels in BHK-V (A) and L929 (B) cells infected with an MOI of 1 with the G3A, G3A-A30C, G3A-C24U and G3 VEEV/SINV VRPs expressing SEAP or RSV-F antigen. Infected cells were analyzed 24 h post-infection. The antigen levels were measured by a chemiluminescent assay (SEAP) or ELISA (RSV-F) on the collected supernatants and normalized for the number of positive cells. (C) Interferon induction in L929 cells, infected with an MOI of 1, measured by IFN-β specific ELISA in the supernatant harvested 24 h post-infection and normalized for the number of positive cells. Each bar represents the average of three (A, B) or two (C) different experiments. Error bars represent the standard error of the mean.

because it could combine the G3A-like specific infectivity and IFN induction with the higher antigen expression typically reported for the G3 backbone. On the other hand the G3A-A30C, which is missing the predicted single-strand RNA tail in the (−) RNA structure, could be helpful in defining the role of the single-strand tail on the promoter function.

To further assess the effects of these mutations in vitro and in vivo, each replicon was packaged into VRPs by providing the SINV capsid and envelope glycoproteins in trans from defective helpers (Perri et al., 2003). VRP stocks were readily produced at high titers (5–10 × 10^7 IU/ml) from all constructs, suggesting that the changes in the 5′UTR did not have significant effects on genome replication or trans-amplification of defective helper genomes. Using these VRPs, the effect of the encoded mutations on antigen expression levels (SEAP and soluble RSV-F) and IFN production in vitro was determined on IFN-deficient cells (BHK-V) and IFN-competent cells (L929). In all cases, the expression of the proteins (SEAP, RSV-F, or IFN) was standardized to the number of replicon-positive cells in the cultures (Fig. 2). The tested VRPs produced similar levels of protein, independently from the 5′UTR sequence (Fig. 2A, B) and predicted folding. There was a difference in the amount of antigen secreted by the two cell lines, with a lower expression in L929 cells. This reduction was more pronounced in the case of soluble RSV-F, whose expression was more reduced in L929 cells when compared to BHK-V cells (Fig. 2B). To examine the effect of the 5′UTR sequence and predicted secondary structures on innate immune activation, the 5′UTR mutations were examined for their effect on the ability of VEEV/SINV VRP to induce IFN-β. The production of murine IFN-β in vitro was measured in the supernatants of L929, 24 h after infection with either the G3 or G3A-derived VRP, and normalized to the number of antigen expressing cells. As shown in Fig. 2C, cells infected with the G3A replicon produced approximately 2-fold more IFN than the wt G3 replicon. This confirmed that in our chimeric replicon the single G3A change in the 5′ UTR causes an increase in the induction of IFN-induced antiviral response. The higher IFN activation observed in the presence of the G3A TC-83-like sequence could explain the higher sensitivity of TC-83 virus to the antiviral actions of IFN-α/β described by White et al. (2001). IFN levels found in the supernatants of L929 cells infected with the G3A-C24U VRP showed similar levels of IFN production to those detected in L929 cells infected with G3 VRP, whereas the cells infected with G3A-A30C VRPs displayed an IFN response similar, or higher when RSV-F is encoded, to that detected in cells infected with G3A VRPs. This phenotype was independent of the antigen tested. The different response given by G3A-C24U and G3A-A30C is likely to be dependent on the different predicted folding of these RNAs.

Based on computer predictions, the only difference between the two mutants, carrying the same TC-83-like G3A sequence, is the secondary structure of the 5′ UTR. The presence of a less stable structure, with multiple bulges and stems, predicted in the G3A and G3A-A30C (−) RNA may be better recognized by the host nucleic acid-sensing machinery that triggers the activation of type I IFN response.

We next examined the effect of 5′ UTR modifications on antigen production in vivo, by following the levels of SEAP in the sera of mice infected with 10^6 IU of VRPs carrying the wt and mutant VEEV/SINV replicons. At 24 hours post-injection the G3 replicon produced levels of SEAP that were significantly higher than the G3A replicon antigen production ($p=0.019$ as determined by a 2-tailed t-test), and this pattern was maintained at 3 days post-inoculation. By 7 days post-inoculation, no differences were detectable. The G3A-C24U and G3A-A30C expression levels were in between the G3A and G3 replicons, with G3A-C24U expression pattern closer to the wt G3 5′ UTR phenotype (Fig. 3A). Interestingly, these in vivo phenotypes confirmed the presence of two classes of mutants, according to their predicted folding. This was in agreement with what we observed in vitro, and much clearer especially in terms of antigen expression.

To assess the immunogenicity of our panel of mutant replicons, mice were immunized with 10^6 IU of the indicated VRP on days 0 and 21. Sera were collected 3 weeks after the 1st vaccination (3wp1) and 2 weeks after the 2nd vaccination (2wp2) and tested for F-specific IgG titers (Fig. 3B). F-specific splenic T cell responses were measured when the animals were euthanized at 2wp2 (Fig. 3C). G3A and G3 VRPs both elicited a strong, F-specific serum IgG response in mice, both after the first and second vaccination, with responses detected in 100% of the treated mice. The F-specific responses detected in the sera of mice inoculated with the VRP carrying the G3A-C24U replicon appeared to elicit stronger responses than those detected in sera from mice inoculated with VRPs carrying the G3A replicon following both vaccinations, but this difference was small ($p=0.045$ at 2wp2 as determined by a 2-tailed t-test). The positive effect on antibody induction of the G3A-C24U replicon (Fig. 3B) could be due to the combination of the slight increase in antigen expression, typical of the G3-like replicon structure at early times post-inoculation (Fig. 3A), and/or the increased specific infectivity conferred by the G3A mutation (Fig. 3C). However, when we applied Kruskal–Wallis test and Dunn ′s post test to account for multiple comparisons none of the differences in antibody titers elicited by the G3A-C24U, G3A and G3 VRPs were statistically significant. F-specific responses detected in the sera of mice inoculated with VRPs carrying the G3A-A30C replicon appeared to be weaker than those detected in sera from mice inoculated with VRPs carrying either the G3 or G3A

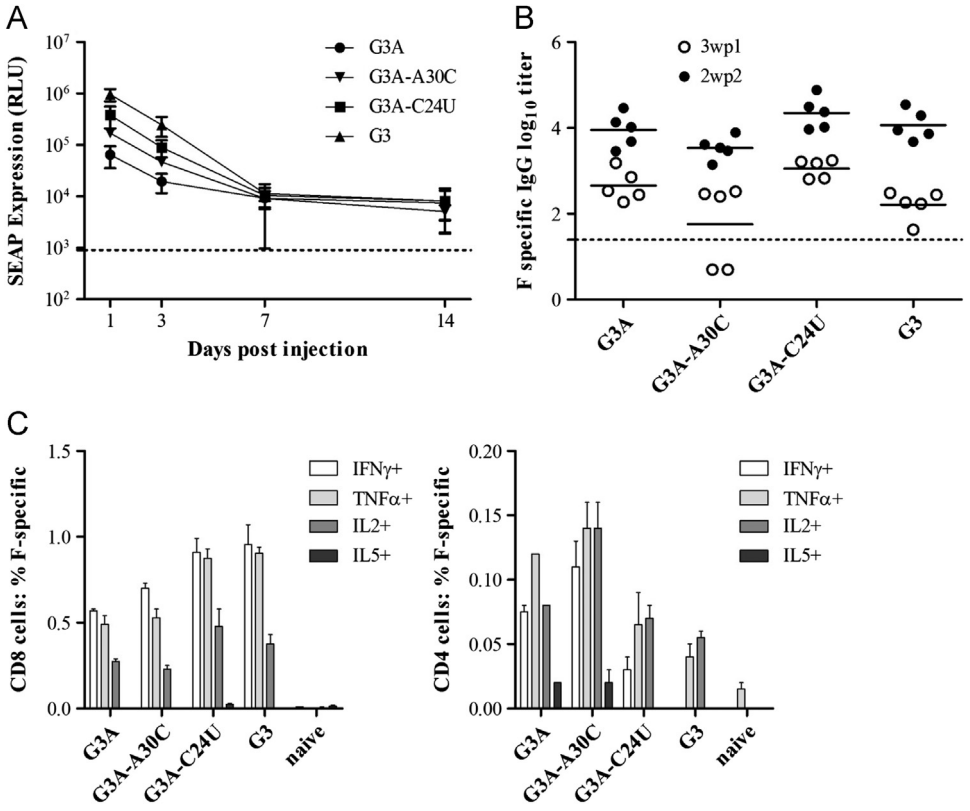

Fig. 3. Effect of 5′ UTR mutations on in vivo antigen expression and immunogenicity of VEEV/SINV replicons. (A) The indicated replicons expressing SEAP were packaged as VRP and injected in BALB/c mice (five per group) at 10^6 IU/mouse. SEAP expression (shown as RLU on a logarithmic scale) was measured in the sera collected at 1, 3, 7, and 14 days post-injection as described in Material and Methods. Average of SEAP expression from five mice/group is represented for each time point and each tested replicon. Error bars indicate the standard deviation. The dashed line indicates the amount of SEAP measured in the sera from mice inoculated with PBS. (B, C) To look at immunogenicity of the 5′ UTR modified replicons, BALB/c mice (five mice per group) were injected with the indicated VRP (10^6 IU/mouse) expressing RSV-F antigen. (B) RSV-F-specific serum IgG titers of BALB/c mice, after intramuscular vaccination with the indicated vaccines on days 0 and 21. Serum was collected for antibody analysis on days 21 (3wp1) and 35 (2wp2). Data are represented as titers for individual animals and the geometric mean titer for each group is shown. The dashed line indicates the limit of titer quantification. (C) Frequencies of RSV-F-specific cytokine-producing $CD4^+$ or $CD8^+$ splenic T cells of vaccinated BALB/c mice. Spleens were collected for T cell analysis on day 35 (2wp2). Shown are average net F-specific cytokine-positive frequencies (%) of duplicate wells of one pool of five spleens per group. Error bars indicate the standard error of the mean.

replicons following both vaccinations. These differences were significant only if comparing the response elicited by the G3A-A30C VRPs to the G3 one at 3wp1 ($p=0.0231$ as determined by Kruskal–Wallis test and Dunn′s post test). The weaker response elicited by G3A-A30C with respect to those of either G3 or G3A VRP at 2wp2 were not statistically significant (as determined by Kruskal–Wallis test and Dunn′s post test).The absence of the single-strand RNA tail in the RNA predicted structure did not alter the promoter function.

Frequencies of F antigen-specific, cytokine-producing CD8+ and CD4+ T cells in spleens of vaccinated mice were measured for the four modified VRPs. G3A and G3 VRPs both elicited a robust F-specific $CD8^+$, and moderate $CD4^+$ T cell response, each producing the type-1 cytokines IFNγ, TNFα, and IL-2, without significant production of the type-2 cytokine IL-5 (Fig. 3C, D). The F-specific T cell responses measured in the spleen of mice inoculated with the VRP carrying the G3A-C24U replicon appeared to be more similar to the G3 responses. On the other hand, G3A-A30C replicon elicited levels of CD4+ and CD8+ T cell response more similar to those elicited by the G3A replicon.

Taken together, these data suggest that the sequence of the very 5′end of the 5′ UTR is a determinant of the immunogenicity of alphavirus replicons and its structure plays an important role in determining their phenotype. Both in vivo and in vitro data showed that replicons carrying a G3A or G3 predicted folding act differently in controlling antigen expression and type I IFN induction, even though these changes did not correlate with large changes in immunogenicity.

Effect of nsP1/2 cleavage site mutations on expression levels, activation of innate immune response, and immunogenicity of VEEV/SINV replicons

To further investigate the role of IFN response on replication, expression and immunogenicity of our replicon vaccine, we decided to modify the cleavage site between nsP1 and nsP2 coding regions, as this site has been described as a major determinant of viral pathogenesis in SINV and RRV (Cruz et al., 2010; Heise et al., 2003). Modification of the P3 position (Thr538Ile in SINV and Ala532Val in RRV) in the cleavage site between nsP1 and nsP2 genes is responsible for an enhanced type I IFN induction and virulence attenuation (Cruz et al., 2010).

To determine if the nsP1/nsP2 cleavage site is involved in the properties of our vaccine replicon, we changed the P3 cleavage position in nsP1 in our G3A VEEV/SINV replicon. The correspondent P3 position in VEEV is in position 533 and encodes an Alanine. We examined whether substitution with an Isoleucine (A533I) or Valine (A533V) or Threonine (A533T) (Fig. 4A) would result in a viable replicon with similar effects on IFN induction, as seen with the SINV T538I and the RRV A532V mutants. All three substitutions of Alanine 533 resulted in replicons that were able to produce VRPs, although all three grew to lower titers (1.5-2 logs lower, data not shown) than the VRPs made with the parental G3A replicon. To evaluate the effect of these mutations on IFN response, L929 cells were infected with VRPs carrying the G3A SEAP replicon or derivatives carrying each of the mutations. This preliminary study showed that all replicons induced more IFN than the

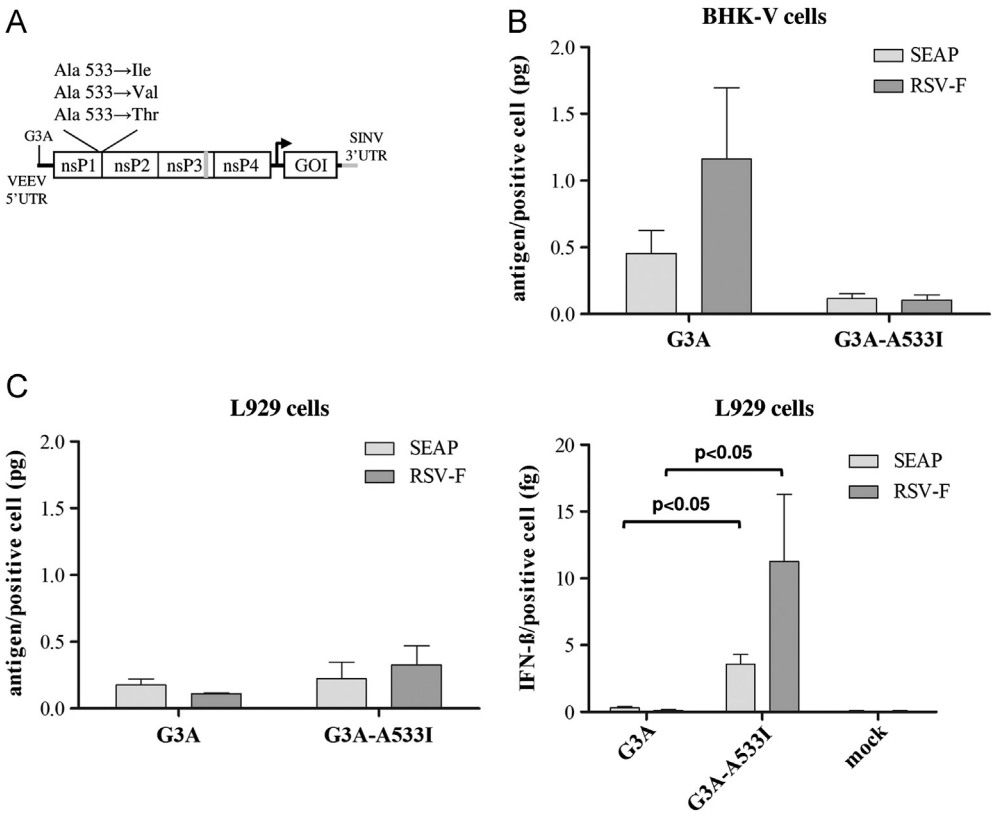

Fig. 4. Effects of nsP1/nsP2 cleavage site modification on antigen expression and activation of innate immune response of VEEV/SINV replicons. (A) Three different VEEV/SINV replicons were cloned starting from G3A genome by mutation of the Alanine in position 533 (P3 cleavage position) in Isoleucine, Valine or Threonine. The selected G3A-A533I mutant was selected for further characterization. G3A and G3A-A533I VRPs were used to infect BHK-V and L929 cells with an MOI of 1. (B) Antigen expression levels were measured 24 hours post-infection in the supernatant coming from BHK-V (B) and L929 (C) cells by a chemiluminescent assay (SEAP) or ELISA (RSV-F). The antigen expression levels were normalized for the number of positive cells. (D) Type I IFN induction was measured in the supernatant of infected L929 cells 24 h post-infection. (B, C, D) Each bar represents the average of three different experiments. Error bars represent the standard error of the mean. p-values were determined by 2-tailed t-tests.

parental G3A replicon, but the highest induction was observed in the cells infected with the A533I mutant (results not shown), so it was selected for all further studies. We cloned the RSV-F antigen in the A533I replicon to investigate the effects of this mutation on replicon properties. We first compared the antigen expression levels in vitro of G3A and G3A-A533I mutants expressing SEAP or RSV-F. The introduction of the A533I mutation resulted in a 2- to 5-fold decrease in antigen expression in BHKV cells (Fig. 4B), but no difference was observed in infected IFN-competent L929 cells (Fig. 4C). Next, we compared the ability of the parental and A533I replicon to induce IFN responses. These studies showed a profound increase in IFN response for G3A-A533I mutants independent of the antigen expressed (Fig. 4D), but the difference was more pronounced with replicon expressing the RSV-F protein. For the pair of replicons expressing this antigen, the A533I mutation resulted in a 15-fold increase in the IFN induction in L929 cells when compared to the G3A replicon ($p=0.02$ as determined by a 2-tailed t-test) (Fig. 4D). These data demonstrate that the effect of nsP1 cleavage mutations on IFN induction by arthralgic alphaviruses also applies to our VEEV/SINV chimera.

To further evaluate this interesting mutation, in vivo studies were conducted comparing the G3A and the G3A-A533I replicons. Fig. 5A shows the studies comparing SEAP expression from mice injected with SEAP-expressing VRP carrying the G3A and G3A A533I mutant replicons. Interestingly, there were only modest differences in SEAP produced from these replicons in vivo (Fig. 5A) ($p=0.02$), although the A533I replicon showed lower antigen expression levels in vitro (in BHK), and higher levels of IFN induction. Next, the G3A and the G3A-A533I RSV-F expressing VRP were used to immunize 6–8 weeks old BALB/c mice. A comparison of the outcome of vaccination using both serological and cellular readouts (Fig. 5B, C), demonstrated that the G3A-A533I replicon was much less immunogenic than the G3A parental replicon, in particular after the first vaccination ($p=0.01$ at 3wp1 as determined by a 2-tailed t-test). These results are consistent with the possibility that the high IFN induction predicted from our in vitro studies with the G3A-A533I replicon (Fig. 4D), and the fact that viruses carrying the G3A mutation are more sensitive to IFN than viruses with a G3 genome (White et al., 2001), combined to make this replicon less immunogenic.

Discussion

Alphavirus replicon-based vectors hold great promise as vaccine delivery vehicles and the VEEV/SINV chimeric replicon described in this report is especially well suited for this purpose. While the potency of alphavirus-derived replicon vaccines has been established in preclinical and clinical studies (see Introduction), little is known regarding the critical interplay between antigen expression and innate immune activation which regulate replicon-induced immunity. In this report we investigated the importance of the replicon genome sequences in controlling the interactions with the host in order to rationally design more potent alphavirus replicon-based vaccines.

The immune response elicited by alphavirus based vaccines is believed to be determined by both high antigen expression (Liljestrom and Garoff, 1991) and innate immune activation (Hidmark et al., 2006; Thompson et al., 2008a). Various steps in the alphavirus life cycle that are either an outcome of the needs for efficient replication and/or interactions with its hosts immune

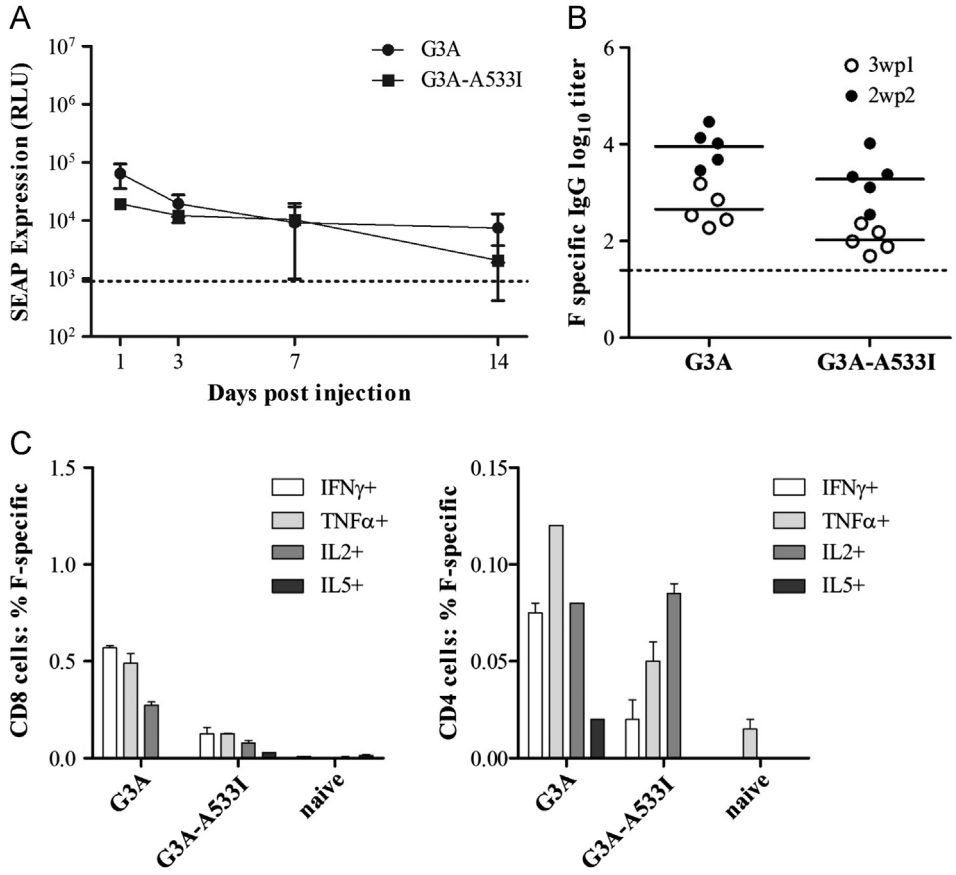

Fig. 5. Analysis of the effects of A533I mutation on in vivo antigen expression and immunogenicity of VEEV/SINV replicons. (A) The differences observed in vitro had limited effects in vivo. Six to eight weeks old BALB/c mice (five per group) were injected with 10^6 IU of the G3A or G3A-A533I VRPs. SEAP expression was measured in the sera collected at 1, 3, 7, and 14 days post-injection as described in Material and Methods. Average of SEAP expression from five mice/group is represented for each time point and each tested replicon. Standard deviation is represented by the error bars. The dashed line indicates the amount of SEAP measured in the sera from mice injected with PBS. (B) RSV-F-specific serum IgG titers, after intramuscular vaccination in mice (five per group) with the indicated vaccines on days 0 and 21. Serum was collected for antibody analysis on days 21 (3wp1) and 35 (2wp2). Data are represented as titers for individual animals and the geometric mean titer for each group. The dashed line indicates the limit of titer quantification. (C) Frequencies of RSV-F-specific cytokine-producing $CD4^+$ or $CD8^+$ splenic T cells of BALB/c mice, after intramuscular vaccination with the indicated vectors. Spleens were collected for T cell analysis on day 35 (2wp2). Shown are average net F-specific cytokine-positive frequencies (%) of duplicate wells of one pool of five spleens per group. Error bars indicate the standard error of the mean.

response undoubtedly regulate these attributes of alphavirus vectors. We applied a genetic approach to analyze a subset of mutations taken from attenuated alphaviruses that could affect these events to try to increase the potency of our alphavirus replicon-based vaccines. We started our analysis by mutating sequences to change both primary and secondary structures within the RNA promoter region of the genome. The VEEV core promoter (5′ UTR) for the positive strand genome synthesis is an important regulator of viral pathogenesis (Kinney et al., 1993), and both primary sequence and secondary structure play a critical role in its function (Kulasegaran-Shylini et al., 2009a). The VEEV TRD genome 5′ UTR (corresponding to the sequence of the G3 replicon used in this study) is predicted to form a stem-loop structure on the (+) RNA strand that is mirrored on the (−) RNA strand. Modification of a single base at position 3 of this promoter, which is found in the attenuated TC-83 vaccine strain, can dramatically change viral pathogenesis (Kinney et al., 1993) and phenotype (Kulasegaran-Shylini et al., 2009b). Previous work has shown that this mutation reduces the stability of the 5′-terminal stem loop on the (+) RNA strand and causes a similar change in the 3′end of the (−) RNA strand intermediate. As a result, this mutation affects multiple processes of the virus replication cycle, changing RNA infectivity, and the transcription of the subgenomic RNA (Kulasegaran-Shylini et al., 2009b).

In the present study, the TC-83-like G3A mutation was transferred to our VEEV/SINV chimeric replicon. This single G3A change in the 5′ UTR caused a decrease of antigen expression (in vivo) and an increase in the induction of IFN-induced antiviral responses (in vitro) with respect to the chimeric replicon carrying a wt 5′ UTR sequence. Using the data from Kulasegaran-Shylini et al. (2009a) and in silico computer prediction of RNA secondary structures (Jaeger et al., 1989) we selected mutations having an A in position 3 and a folding resembling the conformation of the G3 or G3A replicon on the (−) RNA strand. By comparing two set of mutants having the G3 or G3A sequence and folding respectively, it was clear that the major determinant of in vitro specific infectivity of transfected RNA replicons was the sequence in position 3, with all the mutants carrying the G3A mutation having a higher specific infectivity than the G3 derived mutants. Based on the available data (Kulasegaran-Shylini et al., 2009a; Kulasegaran-Shylini et al., 2009b), we expected to see two classes of mutants regarding the expression of the antigen, with a higher subgenomic transcription driven by the G3 5′ UTR. Analysis of antigen production in vivo after injection of replicons delivered as transpackaged VRP showed an expression pattern that seemed to be regulated by the predicted folding, with the replicons carrying the G3-like folding expressing more SEAP in vivo, independently of the nucleotide sequence at position 3.

Analysis of the effect of these mutations on the replicon's ability to induce IFN response when delivered as VRP in IFN-competent cells showed an interesting pattern. It has widely been described that the host nucleic acid sensing machinery that

triggers the activation of type I IFN response better recognizes double strand RNAs, which activate the RNA helicase RIG-I to initiate expression of IFN α/β (reviewed in Ryman and Klimstra, 2008). Among the panel of mutants we tested, the G3A-A30C had both the G3A sequence and the TC-83 like predicted folding on the (−) RNA strand and was able to induce higher level of IFN, with respect to the G3 control. This phenotype, similar to the G3A one, could be explained by the presence in the predicted secondary structure on the (−) RNA strand of a dsRNA stem longer than the one predicted in G3 wt genome and of a smaller terminal loop. A further confirmation of the role of the secondary structure on IFN activation came from the analysis of the G3A-C24U mutant. This 5′ UTR sequence which has been previously described (Kulasegaran-Shylini et al., 2009a), is characterized by the TC-83-like G3A mutation and by a predicted stem-loop structure that is identical to the G3 5′ UTR on both the (+) and (−) RNA strands. In our IFN induction assay the VEEV/SINV replicon carrying the mutated G3A-C24U 5′ UTR was similar to the G3 5′ UTR in terms of eliciting IFN secretion from infected cells.

The precise effect of IFN activation on immunogenicity of alphavirus based vaccines is difficult to predict, since IFN induction could have either a beneficial or a detrimental effect on the development of the antigen-specific immune response. The intrinsic adjuvant activity of the replicating RNA in the infected cells, through the recognition by the nucleic acid-sensing machinery, may potentiate antigen-specific adaptive immune responses via IFN induction. A specific role for type I IFN signaling in alphavirus-induced adaptive immunity has previously been established as it has been shown that the activation of B and T lymphocytes is significantly impaired in IFN α/β receptor knock-out mice (Alsharifi et al., 2005). Additional studies performed by Leitner and co-workers suggested that the ability of replicase-based vaccines to break immunological tolerance was dependent upon a single interferon stimulated gene involved in the viral dsRNA response, RNaseL (Leitner et al., 2003). These early studies are consistent with more recent studies demonstrating that alphavirus replicon-based vaccines induce IFN and can adjuvant co-delivered antigens (Hidmark et al., 2006; Thompson et al., 2008b).

On the other hand, early type I IFN activation could lead to inhibition of RNA replication and to a reduction of antigen expression. In particular, there are reports that alphavirus vector-expressed antigen levels are increased in animals lacking a functional IFN receptor (Leitner et al., 2006; Ryman et al., 2002), suggesting that, in wt mice, autocrine and paracrine IFN signaling limit VRP antigen expression.

In our study, the differences in IFN induction measured in vitro among the replicons carrying different 5′ UTR sequences did not produce dramatic differences in the immunogenicity of the tested replicons. However the replicon which induced the highest amount of IFN in our in vitro system, G3A-A30C, appeared less potent in vivo than other replicons, suggesting that the IFN response it generated was detrimental to induction of an antibody response to its encoded antigen.

To further investigate this detrimental effect of IFN induction on replicon potency in vivo, we engineered the genome of our replicon to increase its ability to induce an IFN response. To this end, we targeted the cleavage site between nsP1 and nsP2 coding regions. Based on reports from two distantly related alphaviruses, the AR86 strain of Sindbis and Ross River virus showing that mutations in this region resulted in enhanced type I IFN induction, loss of STAT1 inhibition and IRF-3 activation in comparison to the wt viruses (Cruz et al., 2010; Heise et al., 2003), we elected to mutate the alanine 533 with an isoleucine in the VEEV/SINV nsP1/nsP2 cleavage site. This mutation led to increased IFN production in vitro, confirming the role in IFN modulation of the nsP1/nsP2 cleavage site in VEEV as well. In vivo, the replicon with this mutation was poorly immunogenic, but since it also produced less antigen in vitro and marker gene in vivo, it is difficult to ascribe its poorer potency to antigen production or IFN induction.

The viral determinants and the mechanisms controlling the balance between the adjuvant and the detrimental effect of type I IFN on alphavirus replicons immunogenicity are still not clear. Our findings are consistent with the hypothesis that a balanced, but not overwhelming IFN response has positive effects on vaccine outcome. The precise mechanism used by VEEV to modulate host response to the infection needs to be better understood to enable the definition of the factors involved in virus-induced immunity as well as the design of new strategies to increase the efficacy of VRP as vaccine vectors.

Materials and methods

Cell line propagation and infection

BHK-V cells were maintained in Dulbecco minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine, and penicillin–streptomycin at 37 °C with 5% CO_2. Mouse fibroblast L929 were maintained in DMEM supplemented with 10% fetal calf serum (FCS), L-glutamine, and penicillin–streptomycin at 37 °C with 5% CO_2. Cell monolayers of approximately 80% confluence were infected with replicon particles with an MOI of 1 in DMEM containing 1% FCS at 37 °C and then analyzed 24 h after the beginning of infection. For each replicon to be analyzed, cells were infected in triplicate and in three different experiments.

Plasmid constructs

The initial replicon genome was the NVD VEEV/SINV chimeric replicon that comprises a VEE-based genome containing a SINV packaging signal and SINV 3′ end (Perri et al., 2003). This replicon was modified by replacing the SP6 promoter with the T7 DNA polymerase promoter and by cloning the gene of interest under the control of the 26S subgenomic promoter. The poly(A) sequence was followed by a hepatitis delta virus ribozyme (Dubensky et al., 1996; Perri et al., 2003), followed by a PmeI restriction site, required for the linearization of the plasmid before an in vitro transcription reaction. The resulting replicon is indicated as G3 in the text and it is the replicon used as backbone to test the selected modifications. G3A replicon differed from G3 by one nucleotide in position 3 (G3A), which was specific for the VEEV TC-83 5′ UTR, and by one nucleotide in position 1696 (C1696A), that was specific for the VEEV TC-83 nsP2 (Kinney et al., 1993). The 5′ UTR mutants were obtained by standard PCR-based mutagenesis of the G3 or G3A replicon using the QuikChange II XL Site-Directed Mutagenesis Kit, according to the manufacturer's instructions (Agilent Technologies). A30G, A30C and C24U derived from the G3 replicon, while G3A-A30G, G3A-A30C and G3A-C24U derived from the G3A replicon.

G3A-A533I, G3A-A533V and G3A-A533T were cloned from G3A by standard PCR-based mutagenesis of nucleotide corresponding to amino acid in position 533 of VEEV. All the modifications were tested in the context of SEAP or RSV-F expressing replicons. RSV-F cDNA encoded for the truncated fusion peptide deleted form of the F antigen of RSV. The cDNA derives from wt F of RSV strain A2, a prototypic laboratory subgroup A strain isolated in 1961 from an infant with bronchiolitis. The RSV-F cDNA used in this study differs from wt F of the A2 strain because of deletion of the N-terminal nine amino acids of the fusion peptide (amino acids 137–145 of the wt F sequence) and of the transmembrane and cytoplasmic tail regions (amino acids 524–574 of the wt sequence).

RNA transcripts

Prior to transcription, the VEEV/SINV genome-coding plasmids were linearized by PmeI. PmeI-linearized replicons were used for in vitro transcription reactions. RNAs were synthesized by T7 RNA polymerase in the presence of nucleoside-5′-triphosphates (NTPs), under the conditions recommended by the manufacturer (Ambion′s MegaScript Kit). Unincorporated nucleotides were removed by Lithium Chloride precipitation and the 7-methyguanylate cap structure (Cap 0) was added to the in vitro transcribed RNA using the Vaccinia Virus Capping Enzyme under the conditions recommended by the manufacturer (CellScript). The capped RNAs were purified by Lithium Chloride precipitation. The yield and integrity of the transcripts were monitored by gel electrophoresis under non-denaturing conditions, followed by analysis of the RNA concentration by OD_{260} of a diluted aliquot of the reaction.

Production of alphavirus replicon particles

Virus replicon particles (VRP) were generated by co-electroporation in BHK-V cells of in vitro-transcribed RNAs, corresponding to a replicon and two defective helpers, one expressing capsid protein and the other expressing envelope glycoproteins (Perri et al., 2003). Supernatants were harvested 16 h laters, centrifuged for 10 minutes at 1500 RPM and frozen at −80 °C in small aliquots. To produce VRP for the in vivo studies, supernatants collected from electroporated cells were pelleted through a 20% (w/v) sucrose/phosphate-buffered saline (PBS) cushion at 30,000 RPM by ultracentrifugation (2 h) to purify viral stocks. Purified VRP were concentrated on 100 kDa Amicon Ultra-15 Centrifugal Filter Units (Millipore), in accordance with the manufacturer′s instructions and 0.1 ml aliquots were frozen at −80 °C. Replicon particle titers were determined by intracellular staining of the expressed antigen and of RNA replication following overnight infection of BHK-V cells with serial dilutions of particles. Infected cells with VRP expressing RSV-F antigen were permeabilized and fixed by using acetone-methanol 1:1 and then stained with the antibodies recognizing RSV-F protein (mouse monoclonal anti-RSV-F antibody, ProSci Incorporated used at 0.1 μg/ml final concentration) or dsRNA intermediates (J2 mouse monoclonal antibody, English & Scientific Consulting used at 0.5 μg/ml final concentration). A peroxidase mouse secondary antibody (Jackson Immunoresearch HRP goat anti mouse IgG (H+L)) followed by substrate incubation (KPL TrueBlue™) was used to detect the positive cells and calculate the VRP titers. The titers of SEAP-expressing VRP were determined by dsRNA staining as described above and by SEAP protein expression staining. To detect SEAP positive cells, infected cells were treated with Brefeldin A (Becton Dickinson) at 1.0 μg/ml final concentration for 2 h at 37 °C, fixed with paraformaldehyde 4% (w/v) in PBS, permeabilized with 0.2% Triton-X100 in PBS and washed with alkaline phosphatase staining buffer (Tris 100 mM, pH 9.5, NaCl 100 mM, MgCl2 20 mM). Cells were incubated overnight with the staining solution (0.1 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, disodium salt (Sigma), 0.5 mg/ml Nitro Blue tetrazolium (Sigma) in alkaline phosphatase staining buffer) and the reaction was stopped with water. The number of SEAP expressing cells was counted and the titer determined.

RNA transfections

The specific infectivity of in vitro-transcribed, capped replicons was measured by RNA transfection (serial dilutions starting from an RNA amount corresponding to 10^{11} copies/well) into BHK-V cells in 96-well plates by using Lipofectamine 2000 (Invitrogen) and Opti-MEM medium (Invitrogen) according to the manufacturer′s recommendations. The transfection mixture was removed after 12 h of incubation and was replaced with DMEM plus 10% FBS. Cells were analyzed at different times after transfection. For each replicon to be analyzed, cells were transfected in triplicate and in two different experiments.

Flow cytometry

To determine the relative RSV-F expression, the viral RNA replication, and the number of positive cells, VRP or mock infected BHK-V and L929 cells were analyzed by flow cytometry. Cells infected with RSV-F expressing VRP were double stained with the antibodies specific for RSV-F protein (mouse monoclonal anti-RSV-F antibody, ProSci Incorporated) and for dsRNA intermediates (J2 mouse monoclonal antibody, English & Scientific Consulting), while cells infected with SEAP expressing VRP were stained with the dsRNA specific antibody. Briefly, 24 h after the beginning of infection, mock and VRP infected cells were rinsed and dry trypsinized and divided into aliquots in parallel groups for staining. Each aliquot was incubated in staining buffer (0.25% BSA, 0.2% NaN3 in PBS), permeabilized and fixed by using a Cytofix/Cytoperm kit (Becton Dickinson). The samples were stained with the primary antibody, previously conjugated with a fluorescent Fab fragment with the Zenon Mouse IgG Labeling Kit (Invitrogen), according to the manufacturer′s recommendations. Parallel cell aliquots were left unstained. Stained and unstained cells were washed and analyzed on a BD Biosciences LSR II flow cytometer. FlowJo software (Tree Star) was used to analyze the acquired data.

Analysis of protein synthesis

Supernatants from infected cells were used to assess the amount of secreted antigen by a chemiluminescent assay (SEAP) or ELISA (RSV-F) 24 h after infection.

The levels of secreted SEAP were measured using the Tropix Phospha-Light System Kit according to the manufacturer′s instructions (Applied BioSystems) in the presence of a standard curve for protein quantification.

To determine the concentration of RSV-F protein secreted by the infected cells a capture ELISA was performed. Plates coated with 0.06 μg/well of monoclonal RSV-F capture antibody (Maine Biotech) were incubated with serial dilutions of supernatants or of a purified RSV-F protein used to create a standard curve. Bound protein was detected with a detection monoclonal antibody to RSV-F, previously conjugated with HRP (Maine Biotech), and followed by substrate incubation and luminometer reading at $OD_{450\ nm}$.

The antigen expression levels measured in the supernatant of in vitro infected cells were normalized for the number of positive cells, as determined by cytofluorimetric analysis or SEAP protein expression staining. Results are reported as pg/positive cell, unless otherwise mentioned. Each sample was tested in triplicate and the shown data are the result of three different experiments.

Type I IFN assay

The levels of murine IFN-α/β in the supernatant from infected L929 cell cultures were measured 24 h post-infection, by using the Mouse Interferon Beta ELISA Kit from PBL Biomedical Laboratories, under the conditions recommended by the manufacturer. The measured levels of IFN-β were normalized for the number of positive cells, as determined by cytofluorimetric analysis. Results are reported as fg/positive cell, unless otherwise mentioned.

Each sample was tested in triplicate and the shown data are the result of three different experiments.

Animal studies

Six to eight weeks old BALB/c mice were obtained from Charles River Laboratories, and were housed under specific pathogen-free conditions, and experiments were approved and performed according to Novartis Institutional Animal Care and Use Committee guidelines.

To measure in vivo protein expression driven by the different replicons, mice (five per group) were injected with SEAP-expressing VRPs at 10^6 IU/mouse (administered intramuscularly 50 μl in each hind leg for mice) on day 0 and sera were collected at 1, 3, 7, and 14 days post-injection. SEAP expression was measured using the Tropix Phospha-Light System kit according to the manufacturer's instructions (Applied BioSystems) in duplicate wells for each mouse at each time point. Average of the two wells was considered for each mouse and average of 5 mice was considered for each time point in the analysis.

Animals were immunized with the indicated RSV-F expressing vaccines on days 0 and 21. Immunizations were performed intramuscularly in 100 μl total volume (administered 50 μl in each hind leg for mice). Serum was collected for antibody analysis 3 weeks after the 1st vaccination (3wp1) and 2 weeks after the 2nd vaccination (2wp2). Mouse spleens were collected at the time of sacrifice (2wp2) for T cell analysis. Endpoint immunogenicity assays include: ELISA to determine mouse F-specific serum IgG titers, intracellular cytokine staining and flow cytometry to determine F-specific splenic T cell responses in mice.

Both for SEAP expression and RSV-F immunogenicity in vivo evaluation, the controls (G3 or G3A) and the described modified replicons were tested side by side in the same animal study.

ELISA for F-specific serum antibody

RSV-F-specific IgG titers were determined by ELISA according to the methods described in Geall et al. (2012).

Intracellular cytokines immunofluorescence assay

The five spleens in each group of mice were pooled, and single cell-suspensions were prepared. Two antigen-stimulated cultures and two unstimulated cultures were established for each splenocyte pool. Antigen-stimulated cultures contained 1×10^6 splenocytes, RSV-F peptides representing amino acid sequences 85–93, 249–258 (each at 1 μM), RSV-F peptides representing amino acid sequences 51–66, 164–178, and 309–323 (each at 5 μM), anti-CD28 mAb, and brefeldin A (BD Biosciences). Unstimulated cultures did not contain RSV-F peptides, but were otherwise identical to the stimulated cultures. Cells were cultured, stained with antibodies specific to CD8 and CD4, fixed and permeabilized, stained with antibodies specific to IFNγ, TNFα, IL2, and IL5, and analyzed according to the methods described in Geall et al. (2012). The net (%) F-specific T cell response was calculated as the difference between the percent cytokine-positive cells in the stimulated cultures and the percent cytokine positive cells in the unstimulated cultures.

Statistical Analyses

Statistical analysis (paired 2-tailed t-test, Kruskal–Wallis test and Dunn's post test) was performed using Prism 5 software (GraphPad).

Computer analysis of the RNA secondary structures

The optimal secondary structures of the RNAs were predicted by using the energy minimization program M-Fold (Jaeger et al., 1989).

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